NOVEL PROTAC-BASED EZH2 DEGRADERS EFFECTIVELY TARGET EZH2-DRIVEN LYMPHOID MALIGNANCIES BY ABOLISHING BOTH METHYLATION-DEPENDENT AND -INDEPENDENT FUNCTIONS

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INTRODUCTION

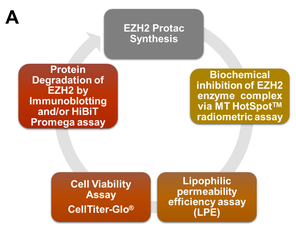
Across various cancers, overexpression or mutation of the epigenetic silencer EZH2 correlates with invasive cancer growth, resistance and poor survival outcomes. While the tumorigenic role of EZH2 is largely attributed to the H3K27me3-mediated repression of tumor suppressor genes, reports have also established a methylation-independent role for EZH2 in the transcriptional activation of oncogenes, particularly in aggressive cancers like Natural Killer T-cell Lymphoma (NKTL) where traditional EZH2 inhibitors have been ineffective.[1,2] We hypothesized that the targeted depletion of EZH2 protein will more effectively disrupt oncogenic signals arising from enzyme-independent activities of EZH2. No clinical EZH2 degraders has been reported yet.

AIM

Design and development of novel EZH2 **Pro**teolysis **Ta**rgeting **C**himeras (**Protac**s) to trigger targeted ubiquitination and proteasome-mediated degradation of EZH2 particularly in aggressive cancers like NKTL.

METHOD

Design, Synthesis and Evaluation of EZH2 Protacs



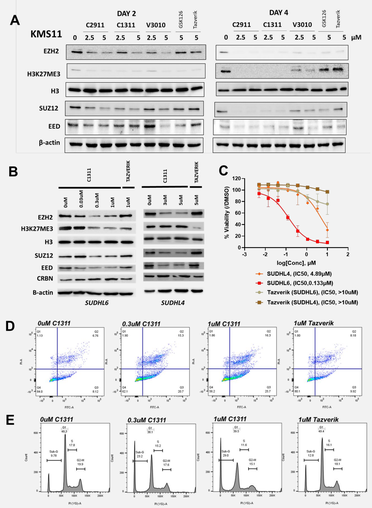
(A) Assays for screening for evaluation and selection of lead EZH2 Protac candidates.

(B) Factors optimized in each series for the iterative synthesis of EZH2 candidates. A total of 53 compounds were screened.

RESULTS

Novel EZH2 Protac induces cell death in DLBCL and NKTL more potently than EZH2 inhibitor

Figure 1. Lead EZH2 Protacs effectively degrades EZH2 protein, PRC2 complex subunits SUZ12; EED and triggers accompanying decrease in H3K27me3 levels in (A) model MM cell line, KMS11. (B) DLBCL (Diffuse large B-cell lymphoma) cell lines SUDHL6 & SUDHL4. **C1311 demonstrates higher cytotoxic activity in DLBCL as compared to clinical EZH2 inhibitor Tazverik**. **It induces** (C) greater loss of cell viability (CTG assay). (D) higher apoptotic cell death, SUDHL6 (Annexin V FITC assay) and (E) increase in percentage of cells in sub G1, SUDHL6 (Cell Cycle assay).



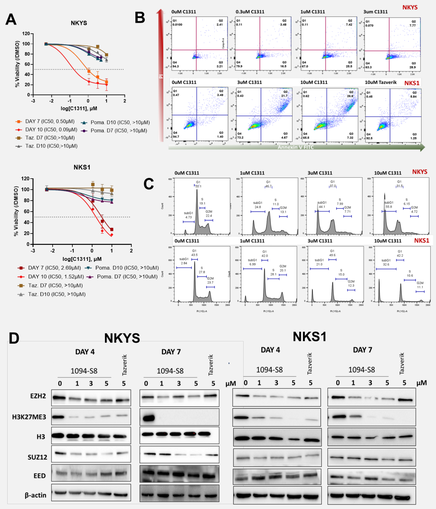


Figure 2. Depletion of EZH2 protein by EZH2 Protac C1311 targets NKTL to cell death whereas treatment with Tazverik displays low efficacy. EZH2 Protac C1311 (A) inhibits cell viability greater than ligand inhibitors leads to dose-dependent increase in apoptosis as measured by (B) Annexin V FITC and (C) cell cycle analysis. (D) C1311 effectively degrades EZH2 protein with the concomitant loss of H3K27Me3 and the PRC2 complex subunit protein levels.

Figure 3. Cycloheximide treatment demonstrates that EZH2 protein has a shorter half life in SUDHL6 than in NKTL cell lines. Cell lines were treated with 0.1mg/ml of cycloheximide at 8h and 18h and the protein levels of EZH2 evaluated. The shorter half life of EZH2 protein in SUDHL6 corresponds with faster EZH2 degradation at day 2 and induction of cell death by day 4 while NKTL cell lines require up to day 4 and day 7 respectively to show those effects. *(figure in next section)*

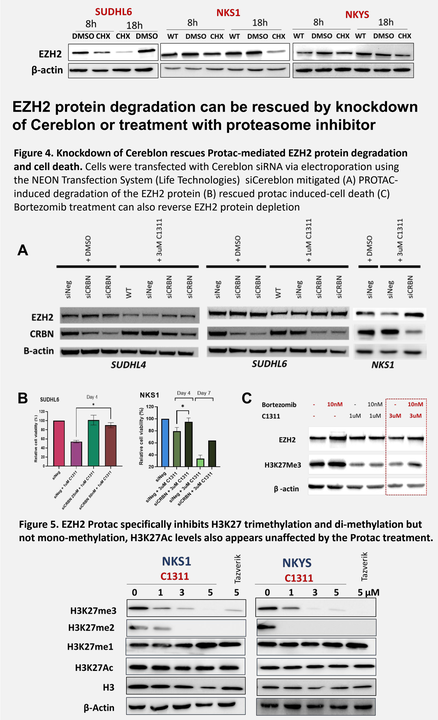


Figure 4. Knockdown of Cereblon rescues Protac-mediated EZH2 protein degradation and cell death. Cells were transfected with Cereblon siRNA via electroporation using the NEON Transfection System (Life Technologies) siCereblon mitigated (A) PROTAC-induced degradation of the EZH2 protein (B) rescued protac induced-cell death (C) Bortezomib treatment can also reverse EZH2 protein depletion

C1311 Protac downregulates the Jak3-STAT5-cMyc axis and demonstrates promising pharmacokinetics *in vivo*

Figure 6. C1311 Protac treatment uniquely disrupts the Jak3-STAT5-cMyc signaling axis in NKTL; this is not achieved by EZH2 inhibitor treatment alone. Immunoblotting detects a decrease in Jak3, cMYC and phosphorylated STAT5 protein levels, suggesting that the Protac may act by disrupting the non-canonical binding of EZH2 to Jak3 in NKTL.

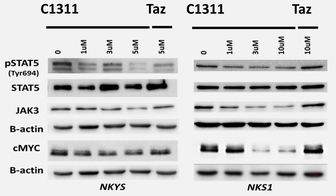


Figure 7. EZH2 Protac treatment increases expression of canonical genes and decreases expression of non-canonical genes. mRNA expression analyses by qRT-PCR revealed that C1311 treatment more effectively reactivated canonical EZH2 target genes such as MYT1 and ANPEP in SUDHL6 and NKTL as compared to Tazverik, while suppressing non-canonical targets such as cMYC and E2F1 in NKTL

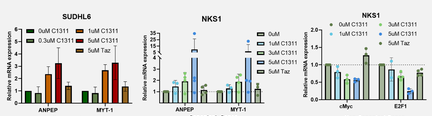
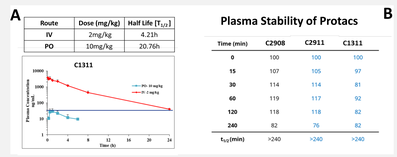


Figure 8 Evaluation of EZH2 Protacs for further clinical development. *In Vivo PK analysis* showed that EZH2 Protacs demonstrate (A) low clearance and decent terminal plasma half-life of up to 4 and (B) good plasma stability (> 4 h),



CONCLUSIONS

The iterative design, synthesis and evaluation of the consecutive series EZH2 Protacs have led to the discovery and development of C1311 as a novel compound which can ubiquitinate and degrade the EZH2 protein in DLBCL and NKTL.

C1311 Protac-mediated depletion of EZH2 and PRC2 protein subunits culminates in the abolishment of both H3K27 trimethylation as well as methyltransferase-independent oncogenic signals of EZH2 altogether enhancing the cytotoxic activity of the Protac beyond that of an EZH2 inhibitor alone.

Treatment with C1311 induces dose-dependent apoptosis, significantly reactivates EZH2 epigenetically suppressed genes such as MYT1 and conversely downregulates oncogenic targets such as cMyc and E2F1 which promote cell proliferation and survival. The corresponding downregulation of Jak3 and downstream STAT5 phosphorylation re-emphasizing the key role Jak3 may play in mediating the enzyme-independent effects of EZH2.[2]

Cycloheximide studies suggest that factors stabilizing protein levels of the Protac protein target may exert a significant impact on the efficacy and duration of EZH2 Protac treatment.

FUTURE WORK

EZH2 Protacs will be tested for anti-cancer efficacy *in vivo*. An in-depth investigation of the mechanisms stabilizing EZH2 protein levels and underlying C1311 induced-cell death will be made to identify potential mechanisms of resistance. This outcomes of these studies can be applied to other EZH2-driven cancers.

REFERENCES

1. **Yan J et al** EZH2 overexpression in natural killer/T-cell lymphoma confers growth advantage independently of histone methyltransferase activity. Blood. 2013 May 30;121(22):4512-20
2. **Yan J et al.** EZH2 phosphorylation by JAK3 mediates a switch to noncanonical function in natural killer/T-cell lymphoma. Blood. 2016 Aug 18;128(7):948-58

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